SCREENING FOR ANTIVIRAL ACTIVITY OF *ACTINOMYCETES* ISOLATED FROM SOIL SEDIMENTS

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Submitted by
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CERTIFICATE

This is to certify that the work embodied in this thesis entitled, **"Screening for antiviral activity of *Actinomycetes* isolated from soil sediments"** submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, was carried by **Mr.S.Kannan (Reg. No. 26073825)**, Department of Pharmaceutical Bio-technology, Nandha College of Pharmacy, Erode-52 for the partial fulfillment for the degree of **MASTER OF PHARMACY** in Pharmaceutical Bio-technology under my supervision.

This work is original has not been previously formed the basis for the award of other degree, diploma, associate ship, fellowship or any other similar title and the dissertation represent entirely an independent work on the part of the candidate.

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The work presented in this thesis entitled **"Screening for antiviral activity of *Actinomycetes* isolated from soil sediments"** carried out by me, under the guidance of**, Prof. Mr. D. KARTHIKEYAN &** Co guidance of **Mrs. R.SUMATHI,** Department of Pharmaceutical Bio-technology, Nandha College of Pharmacy, Erode-52. This work is original & has not been submitted in part or full for the award of other degree or diploma of any other university.

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CONTENTS

1. INTRODUCTION

DEFINITION

Actinomycetes are attractive, bodocious and charming filamentous gram positive bacteria.[1]

Actinomycetes are gram positive bacteria which comprise a group of branching unicellular microorganism. They produce branching mycelium which may be of two kinds viz., substrate mycelium and aerial mycelium.[2]

DESCRIPTION

The search for antibiotics continues to be of extreme importance in research programs around the world because of the increase of resistant pathogens and toxicity of some used antibiotics. The history of new drug discovery possesses shows that novel skeleton have, in the majority of cases, come from natural sources. This involves the screening of microorganisms and plant extracts. Among microorganisms, actinomycetes are one of the most attractive sources of antibiotics and other biologically active substances of high commercial value and from which, *Streptomyces* species has been the most fruitful source of all types of bioactive metabolites that have important applications in human medicine as antiviral and anticancer compounds. Thus, screening and isolation promising strains of actinomycetes with potential antibiotics still a thrust area of search by our group many years.[56]

Actinomycetes are widely distributed in natural and man made environments, and it plays an important role in the degradation of organic matter. They are also well known as a rich source of antibiotics and bioactive molecules, and are of considerable importance in industry. For the purpose of screening novel bioactive molecules, several factors must be considered choice of screening, source, pretreatment, selective medium, culture condition, and recognition of colonies on a primary isolation plate.[4]

Screening of microorganisms for the production of novel antibiotics has been intensively pursued for many years by scientists. Antibiotics have been used in many fields including agriculture, veterinary and pharmaceutical industry. Actinomycetes have the capability to synthesize many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic and enzymes like cellulose and xylanase used in waste treatment.[57]

In the last decades actinomycetes became the most fruitful source of antibiotics. In the 60s and 70s of the 20th century 75-80% of all discovered antibiotics derived from the order actinomycetales mainly from *streptomyces* species. In the 70s and 80s the ratio and significance of the other, non stereptomycete actinomycetes (so called rare actinomycetes) increased up to 20% of all microbial antibiotics and 30-35 of *actinomycetales* species.[5]

Considering the practically useful compounds, today about 130 to 140 microbial products and a similar number of derivatives (including semisynthetic antibiotics) are applied in human medicine, mostly in chemotherapy and vertinary medicine. The majority of these compounds, except fungal *penicillins*, *cephalosporins* and several bacterial peptides and few others, are also produced by actinomycetes. The high percentage of new compounds derived from new target oriented screening method is also of actinomycetal origin. *Amycolatopsis* and *actinomadura* species frequently produced vancomycin-type glycopeptides. The promising antitumor enediyne antibiotics were produced exclusively by rare actinomycetes. The members of new groups of macrolactum and napthacene-quinone antibiotics were isolated from *Actinomadura*. The species from this genus frequently produced polyether antibiotics *Micromonospora* and *Saccharopolyspora* strains were relatively rich sources of Macrolides.[6]

In recent years marine microorganisms have become important in the study of novel microbial products exhibiting antimicrobial, antiviral, antitumour as well as anticoagulant and cardioactive properties. These active compounds may serve as model systems in the discovery of new drugs. *Halophylic* species have been described as *Actinopolyspora halophila*, *Actinopolyspora mortivales* and *Actinopolyspora iraqiencies*. During the source screening for antibiotics from actinomycetes *halophilic Actinopolyspora* species was isolated from alibag a coastal region in the west coast of India.[7]

Both *in vitro* and *in vivo* screening for antiviral substances has long been conducted in our laboratory with *Streptomyces* culture filtrates. Myxoviromycin, kikumycin and formycin were discovered and studied in this continuing effort to develop new antiviral agents. However, with all of these compounds, the concentration in blood after intraperitoneal injection into experimental animals was not high enough to inhibit virus growth *in vitro,* even though a chemotherapeutic effect against certain virus infections *in vivo* can be demonstrated. When the bioretention of the *Streptomyces* culture filtrate reported here was tested by measuring the antiviral effect after intraperitoneal injection in to mice, serum specimens obtained after 10 or 30 minutes were strongly inhibitory to the growth of polio virus and vesicular stomatitis virus.[34]

In the course of an antiviral antibiotic screening program utilizing a paper – disc agar diffusion technique involving Newcastle disease virus (NDV) Miyadera strain with a primary culture of chick embryo fibroblast (CEF), it was found that 62 strains of unidentified *Streptomyces* obtained from soil samples produced cytotoxic and antiviral antibiotics.[29]

Microorganisms isolated from several Zairian soil samples screened for the antiviral activity utilizing the bacteriophage B and *Streptomyces griseus,* regulation of mammalian cell growth and differentiation: detransformation of temperature-sensitive. Rous sarcoma virus – infected rat kidney cells, src^{ts} –NRK; inhibition of mitogenic activity of epidermal growth factor (EGF) on epidermal keratinocytes, Balb-MK cells; induction of granulocyte differentiation of HL-60 human promyeloid leukemia cells.[48]

Isolation methods are usually applied to soil samples or other specimens in a random way without and prior knowledge of the microbial composition of the source under investigation. Consequently, the inability to isolate a certain group of actinomycetes from a given soil, for example can be due to either absence from that soil, or to the use of appropriate methods to recover them, or to their competition by other fast growing bacteria. It would be highly desirable to know in advance what group of microorganisms are present in a natural source, their relative abundance and possible their diversity, so that an appropriate effort can be devoted to that source. The number of bacteria present in a soil sample can be extremely large up to100 g^{-1} of soil. One important factor in increasing the probability of finding novel metabolites is through the use of different strains. While each actinomycetes strain has probably the genetic potential for producing 10-20 secondary metabolites, the probability of finding different metabolites is substantially higher by fermenting different strains than by repeated fermentations of the same strain.[8]

True actinomycetes are bacteria that grow in the form of mycelia, their natural occurance mostly restricted in the soils. They are gram positive and are related to the coryneform bacteria and mycobacteria by an almost continuous sequence of intermediate forms. The name of this group is derived from the first anaerobic species *actinimyces bouis* causes actinomycosis the "ray fungus disease" of cattle.[9]

Today, the production of bioactive metabolites by microorganisms and their antibiotic effect against pathogenic microbes are continuing to attract scientific and public interest. Though almost 20,000 microbial metabolites and approximately 10,000 plant products have been described so far. Secondary metabolism appears as an in exhaustible source of new antimicrobials, antivirals, antitumour drugs. Therefore, continuous efforts are being made in the research on microbial secondary metabolites, which have potential applications such as proteopolysaccharides.[58]

They divide by binary fission and may or may not produce external spores. By far the majority of these microorganisms are soil and water saprophytes (organisms living on decaying organic matter) and are exceedingly important for their roles in the cycles of nature, such as decomposition of organic material and fixation of nitrogen. Actinomycetes can be cultivated easily on simple media and can be identified by their growth on the surface of agar and their fermentation mycelia, substrate mycelia, spores and sporongia.[9]

Oil called geosmin can be isolated from *streptomyces griseus* and is responsible for this odour. It is a 1, 10 dimethyl 1-9decalol. Many *streptomyces* degrade cellulose, chitin and other recalcitrant natural substances. One cellulose degrading organism, widely distributed in soil and in rotting aqueous sediments, is *Micromonospora.* It has flat colonies with no aerial mycelia and its spores occur singly at the end of weakly branched *sporophpres*. *Microbispora* is morphologically similar but produces aerial mycelia and paired conidia.[9]

Several actinomycetes actinoplanes, streptosporangium, ampullariella do not produce spores directly on the aerial mycelia, but in sporangia *streptosporangium* is a cellulose degrading aerobic streptomycetes, on solid media it grows at first by substrate mycelia but forms aerial mycelia at later. The tips of aerial mycelia enlarge and forms spherical sporangia 5-8µm in diameter stage. These can reach to18µm at maturity. The supporting hyphae grows into the spherical end cell where it assumes a helical form and pinches off *sporangiospores.* In *streptosporangium* these are non motile.

. The spores of actinomycetes are generally not heat-resistant but they can withstand dehydration. The only actinomycetes that form heat resistant spores is *thermoactinomyces vulgaris*. This thermophilic and occurs as part of the bacterial flora of damp hay stacks and piles of organic wastes, where heat is generated in their structure and dipicolinic acid content these spores resemble the endospores of bacillus and clostridium.[9,10]

In the last few years, more and more efforts have been made by pharmaceutical companies and university research centers to find safe and efficient drugs for the treatment of viral respiratory diseases, herpes virus infections, and in retroviral infections. New types of antiviral compounds have been discovered and novel more potent and selective derivatives of known compounds have been synthesized. The screening of natural products from microorganisms, plants and algae etc.,[11]

Although actinomycetes constitute only < 0.05% of the cultural microbial community, they have provided many important bioactive compounds and many of them have exhibited anti-viral activity.[12]

Proteinase inhibitors are frequently found in streptomyces species. The main function of proteinase inhibitors regulation of the of endogenous protease activity, but probably they have another function like alkaline phosphates. There is therapeutic experience with aprotonin and ε-amino caproic acid in the marine experimental influenza viral infection.[13]

Actinomycetes produce a diverse array of antibiotics including aminoglycosides, anthrocyclins, glycopeptides, ß-lactams, macrolides, nucleotides, peptides, polyenes, polyethers and tetracyclins.[14]

Compounds reported to have antiviral properties include the didemnins, which are cyclic depsipeptides isolated from *Tridid*emnum species (tunicates). Didemnin B as well as being antiviral, and also shows pronounced antitumour activity.[15]

Many synthetic compounds have been found to show antiviral activity since the finding of cytopathic effect caused by virus infection. However, a few antiviral antibiotics were obtained using tissue culture techniques for a screening. Gliotoxin, tenuazonic acid trichothesin, verrucarrinA and brefeldin. A were reported to show antiviral activity *in vitro* among fungal metabolites.[44]

In a systemic search for microbial metabolites effective against herpes simplex virus type I (HSV-1), a bacterial strain No.M937-B1 isolated from soil samples collected near Lake Yamanaka, was found to produce a complex of new acylpeptide antibiotics, pumilacidin. Pumilacidin exhibited antiviral activity against HSV-1-KOS strain.[43]

A new antibiotic substance with an antiphage activity was obtained from the culture filtrate of *Streptomyces* species No. A-2127, an organism isolated from soil sample collected at Musashikoganei-city, Japan. This substances possesses strong inhibitory activity against various phages including T-series *Escherichia coli* phages, λ phage and *Bacillus subtiles* phage SP-10.[45]

1.3 MORPHOLOGY AND CULTURAL CHARECTERISTICS

Actinomycetes resembles to mold and bacteria

As mold is concerned

The colony is dry, tough, wrinkled covered with aerial mycelium. Mycelium is septate and branched.[9]

As Bacteria concerned

These are having prokaryotic nuclei, the cell wall contain muramic acid and diaminopimelic acid. They are susceptible to antibacterial antibiotics, mycelial filament get fragmented into bacillary and cocoid form.[9]

Identification

1. Aerial Mass Colour

The colour of the mature sporulating aerial mycelium is recorded in a simple way (White, grey, red, green, blue and violet). When the aerial mass colour falls between two colour series, both the colours are recorded.[2]

2. Melanoid Pigments

The grouping is made on the production of melanoid pigments (i.e. greenish brown, brownish black or distinct brown, pigment modified by other colours) on the medium.[2]

3. Reverse Side Pigments

The strains were divided in to two groups, according to their ability to produce characteristic pigments on the reverse side of the colony, namely, distinctive (+) and not distinctive or none (-). In case, a colour with low chroma such as pale yellow, olive or yellowish brown occurs, it is included in the latter group (-)**.**[2]

4. Soluble Pigments

The strains are divided into two groups y their ability to produce soluble pigments other than melanin namely, produced (+) and not produced (-). The colour is recorded (red, orange, green, yellow, blue and violet).[2]

5. Spore Chain Morphology

The species belonging to the genus *Streptomyces* are divided into three sections namely rectiflexibles (RF), retinaculiaperti (RA) and Spirales (S). Spore morphological characters of the strains can be studied by inoculating a loopful of one week old cultures into 1.5% agar medium contained in test tubes at 37ºC. The actinomycete should be suspended and thoroughly mixed in the semisolid agar medium and 1 or 2 drops of the medium could be aseptically pipetted on to a sterile glass slide. A drop of agar should be spread well on the slide and allowed to solidify in to a thin film so as to facilitate direct observation under microscope. The cultures should be incubated at 28 ± 2ºC and examined periodically for the formation of aerial mycelium, sporephore structure and spore morphology.[2]

6. Spore surface

The mature spores of the strain should be carefully placed on the surface of the adhesive tape and gold coating should be applied for half an hour and the specimen can be examined under the electron microscope in different magnifications. The spore silhouettes can be characterized as smooth, spiny, hairy and warty.[6]

1.4 CLASSIFICATION OF ACTINOMYCETES [16]

Actinomycetes can be classified as aerobic actinomycetes and anaerobic actinomycetes.

AEROBIC ACTINOMYCETES[16]

The aerobic actinomycetes can be further classified as

A) Nocardia

B) Actinomadura

C) Dermatophilura

D) Streptomyces

ANAEROBIC ACTINOMYCETES[16]

The anaerobic actinomycetes can be further classified as,

A) Actinomyces

B) Arachi

C) Bifido bacterium

D) Rothia

1.6 ISOLATION OF ACTINOMYCETES. [1]

It was mainly by accident that fleming discovered the mold *penicillium chrysogenum* produces penicillin. The essential steps in searching for a new antibiotic are as follows.

1. Obtain samples of soil, air, plants, vegetables, or water from a variety of environments. Isolate the microorganisms from these samples in pure culture.
2. Test each pure culture against a variety of microorganisms to see whether there is any inhibition or killing of the test organisms.
3. Select any pure cultures that show antimicrobial activity for further study. Grow the cultures to produce a large amount of antibiotic, concentrate the antibiotic material. Perform tests to determine that the antimicrobial activity is due to a new substance, not a previously described antibiotic.
4. Test the material for toxicity and determine its antiviral spectrum.
5. If the antibiotic appears to be a new one, grow large cultures, harvest the antibiotic material and purify the antibiotic.
6. Test further to determine chemical properties and if the antibiotic qualifies as a chemotherapeutic agent by meeting most of the criteria for ideal chemotherapeutic agent. The antibiotic must be non toxic for animals and humans.

For Isolation of actinomycetes strains various recommended media were used, i.e.,

a. Glucose – yeast extract malt agar medium.[17]
b. Luria- Bertani agar medium.[18]
c. Inorganic salt agar medium.[3]
d. Sucrose – nitrate agar medium.[19]
e. Peptone – yeast extract iron agar medium.[19]
f. Glycerol – asparagines agar medium.[19]

g. Oatmeal agar medium.[19]

h. Humic – vitamin agar medium.[20]

i. AV agar medium,[3]

j. Starch casein agar medium.[3]

k. Glycerol – yeast extract medium.[1]

l. Solid – boiled bran barley medium.[21]

m. Olsons medium.[22]

n. Complex solid medium.[23]

o. Minimal medium.[24]

p. Basal mineral salt medium.[25]

1.7 PRESERVATION.

The preservation methods are similar to that of bacteria such as subculturing, freezing especially in liquid nitrogen, freeze-drying and maintenance of strains in mineral oil.[2]

1.8 FERMENTATION

The anaerobic oxidation of compounds by the enzyme action of microorganisms, neither gaseous oxygen not a respiratory chain is involved in this energy yielding process. An organic compound is the electron acceptor.

The term fermentation has been used in a strict biochemical sense to mean the energy production process in which organic compounds act as both electron donors and terminal electron acceptors.[10]

1. The range of fermentation processes.

There are five major groups of commercially important fermentations.

1. Those that produce microbial cells (or biomass) as the product.
2. Those that produce microbial enzyme.
3. Those that produce microbial metabolites.
4. Those that produce recombinant products.
5. Those that modify a compound which is added to the fermentation the transformation process.[10]

2. Media for fermentation process.

All microorganisms require water, sources of energy, carbon, nitrogen, mineral elements and possibly vitamins plus oxygen if aerobic, on a small scale. It is relatively simple to diverse a medium containing pure compounds, but the resulting medium although supporting satisfactory growth may be unsuitable for use in a large process.

On a large scale one most normally use sources of nutrient to create a medium which will meet as many as possible of the following criteria.

1. It will produce the maximum yield of product or biomass per gram of substrate used.
2. It will produce the maximum concentration of product or biomass.
3. It will permit the maximum rate of product formation.
4. There will be the minimum yield of undesired products.
5. It will be of a consistent quality and be readily available through out the year.
6. It will cause minimal problems during media making and sterilization
7. It will cause minimal problems in other aspects or the production process particularly aeration, agitation, extraction, purification and waste treatment.[10]

3. The recovery and purification of fermentation product

Ideally one is trying to obtain a high quality product as quickly as possible at an efficient recovery rate using minimum plant investment operated at minimal costs. Unfortunately, recovery costs of microbial products high as 70% of total manufacturing costs. Obviously, the chosen process and therefore its relatively costs will depend on the specific product.

The choice of recovery process is based on the following criteria.

1. The intracellular or extra cellular location of the product.
2. The concentration of the product in the fermentation broth.
3. The physical and chemical properties of the desired products.
4. The minimal acceptable standard of purity.

5. The magnitude of biohazard of the product broth.
6. The impurities in the fermenter broth.
7. The marketable price for the product.[10]

1. Removal of microbial cells and other solid matter

Microbial cells and other insoluble materials are normally separated from harvested broth by filtration or centrifugation.

a) FILTRATION

Filtration is most common processes used at all scales of operation to separate suspended particles from liquid or gas. Using porous medium which retains the particles but allows the liquid or gas to pass through. It is possible to carry out filtration under a variety of conditions but a number of factors will obviously inflexed the choice of the most suitable type of equipment to meet the specified requirement at minimum overall costs including.

1. The properties of the filtrate, particularly its viscosity and density.
2. The nature of solid particles, particularly their size and shape, the size distribution and packing characteristics.
3. The solid liquid ratio.
4. The need for recovery of the solid or liquid fraction or both.
5. The scale of operation.
6. The need for batch and continuous operation.
7. The need for aseptic conditions.
8. The need for pressure or vacuum section to ensure an adequate flow rate of the liquid.[10]

b) CENTRIFUGATION

Microorganisms and other similar size particles can be removed from the broth by using a centrifuge when filtration is not satisfactory separation method.

Although a centrifuge may be expensive when compared with a filter it may be essential when.

1. Filtration is slow and difficult.
2. The cells or other suspended matter must be obtained free of filter aids.

Continuous separation to a high standard of hygiene is required.[38]

1.9 Extraction and purification of microbial DNA from actinomycete strain.

The DNA extraction procedures can involve cell extraction or direct lysis depending on whether or not the microbial cells are isolated from their matrix.

An extraction protocol generally comprises three steps,

1) Cell Lysis
   i. Chemical
   ii. Mechanical
   iii. Enzymatic
2) Removal of cell fragments and nucleic acid precipitation

3) Purification

1. Cesium chloride density gradient ultra centrifugation.
2. Chromatography.
3. Electrophoresis.
4. Dialysis and filtration.
5. Combined methods.

1) DNA Extraction

All the methods developed for extracting soil microbial DNA are two types. Cell extraction methods and cell lysis methods. Cell extraction depends upon the isolation of microbial cells from their environmental matrix, prior to lysis to release DNA. These methods includes successive cycles of blending and differential centrifugation to recover the intact microbial cells.

Limitations of cell extraction

It is time consuming and that only few samples can be processed in parallel.[39]

a) Direct lysis

Direct lysis methods inspired by the work of Ogrom et al (1987) and Stefen et al (1988) does not require cell isolation. These methods are more often used than cell extraction methods, because of their better recovery more over the extracted DNA seems to be representative of the microbial community of the sample, because a greater number of microorganisms are subjected to the lysis, notably those sorbed on to soil organo mineral aggregates.

Limitations of direct lysis

The major disadvantage of direct lysis is that the method also extracts other organic soil components, such as humic acid and phenolic substances, since these contaminants can disturb or prevent subsequent molecular analysis, a purification step is required.

The efficiency of a microbial DNA extraction from soil also depends on soil quality, particularly on its clay and organic matter contents. Every extraction of DNA from environmental samples requires the following steps.

a. Cell lysis.

b. Removal of cell fragments.

c. DNA purification [39]

b) Cell Lysis:

Cell lysis is generally a particularly critical step in soil DNA extraction. It is designed to release the DNA by breaking the cell wall and membranes of the microorganisms. The cell lysis may be chemical, enzymatic or mechanical. The cell lysis generally combines detergents and lytic enzymes. The most widely used detergent is sodium dodecyl sulfate (SDS), but sarkosyl is some times used. The detergent can be used cold or warm to limit the extraction of a humic substallcer. Lysozyme is the most commonly used enzyme for neuropeptide hydrolysis a polysaccharide component of the bacterial cell wall some proteases, like proteinase k, aehromopeptidase and pronase can also help to free nucleic acids.[39]

Soil samples for which neither lysis by detergent nor enzymatic digestion gives satisfactory yields are treated mechanically, cells could be refractory to chemical or enzymatic lysis because of their, extremely resistant cell wall as for gram positive bacteria or of their in accessibility due to protection of soil components.[40]

The mechanical treatment is more effective and less selective than chemical lysis. Thermal shock, bead mill homogenization, bead beating, microwave heating or ultrasonication have all been used but the simplest method is grinding in liquid nitrogen using mortal pestle or homogenizing with beads of various nature,(Silica, agate,glass) size, quantity.[41]

Thermal shock consist of freezing and thawing samples. The number of freeze thaw cycles and the incubation time in liquid nitrogen or on ice and in a water bath at 50, 65 or 100°C can vary. Thermal shock is less violent than mechanical treatment, ultrasonication is an efficient means of releasing bacteria attached to soil aggregates. Microwave heating is very effective for gram positive bacteria and call also be used for lysing bacteria in soil samples but heating must be moderate because nucleic acid can be damaged. Depending upon the lysis protocol used the size of DNA released varies from 0.1-0.5 to 10 or 20 kbp.[41]

c) Removal of cell fragments and nucleic acid precipitation.

Many investigators has used classical deproteinisation in organic solvents, phenol, phenol – chloroform isoamyl alcohol. The proteins can also be salted out using saturated salt solutions, either sodium chloride, potassium chloride, ammonium acetate or sodium acetate. The proteins precipitate during the centrifugation at low speed and the nucleic acids are recovered in supernatant.[39]

Nucleic acids are concentrated by precipitating them with alcohol, isopropanol, or polyethylene glycol (PEG), PEG or isopropanol can be used instead of ethanol and so reduce the total volume of the sample. (10.54vol- of isopropanol or 0.5vol of PEG instead of 2.5 vol of ethanol. [40]

1.10 PURIFICATION OF DNA

It is often more difficult to purify microbial DNA from farming soil than other environments like water. Because the humic acid and phenolic substances are difficult remove. The several purification methods used are.

a. Cesium chloride density gradient ultracentrifugation
b. Chromatography.
c. Simple dialysis filtration.
d. Combined methods.

a) Cesium Chloride Density Gradient Ultra Centrifugation

Cesium chloride (CsCl) density gradient ultracentrifugation was used in some of the first molecular studies on microbial DNA from soil. The CsCl and ethidium bromide are added samples and the various components of the lysate, i.e. Proteins, polysaccharides, chromosomal or plasmid DNA, RNA, move to stable zones of the density gradient formed by CsCl during the centrifugation according to their density. However this procedure is labor- intensive and time consuming the samples may have to be centrifuged at 150,000 rpm for 48 hrs and this method does not always give clean sample and the method gives large amount of DNA loss.[39]

b) Chromatography

Various chromatographic methods used to purify the crude DNA extract. Size exclusion chromatography (gel filtration) involves separating molecules according to their molecular weights, the gel filtration medium of the microspin column is a porous gel, either sephadex G50, sephadex G75, gel, sephadex G200 or sepharose 4B, molecular larger than largest pores of the gel, those above the exclusion limit like DNA, can not enter the gel and thus are eluted first. Similar molecules enter the pores various extents depending on their size and shape and are eluted later. Chromatography on ion-exchange columns may be selectively bind or elute DNA according to pH and ionic strength. The losses measured are about 20-30% but contaminants often remains and further purification is necessary.[39]

c) Electrophoresis

The contaminants such as humic acids can be removed by electrophoresis. In this technique the standard agarose is used which will be mixed with 0.1mg of ethidium bromide to visualize the DNA separated by electrophoresis. Due to its fluorescence capacity, DNA bands can use visualized as brown bands. Sometimes polyacrylamide is also used for separation of DNA. Purification can be achieved by using the same.[40]

Water – soluble polyvinyl pyrrolidone (PVP) is some times incorporated in to agarose to prevent the migration of humic materials with crude DNA. So its use should be minimized.[41]

c) Dialysis and Filtration

Dialysis was used by Romanowski et al. (1992,1993) to clean DNA extracted from soil micro concentrators, mini columns containing filtration membranes, have replaced classical dialysis. The membrane of micro concentrations has specific molecular weight limits, i.e. they retain the molecule above a specified molecular weight.[39]

d) Combined Methods

In a single purification step we can never achieve 99.99% pure DNA sample so many investigators have combined several purification procedures. The protocol is chosen depending upon its rapidity, its facility and its recovery. Smalla et al (1993) subjected their crude DNA extract to a three step purification procedure,

1) CsCl purification
2) Potassium acetate precipitation
3) A purification step with glass milk.[39]

1.11. Potential of actinomycetes.

- Actinomycetes are most widely distributed group of microorganisms in nature they are attractive, bodacious, and charming filamentous gram positive bacteria.
- They make up in many cases especially under dry alkaline conditions, large part of the microbial population of soil.[1]
- Actinomycetes are filamentous bacteria they are of great importance in a biotechnological processes because of their ability to produce large number of antibiotics and other bioactive metabolite.
- As with every screening programme, the probability of finding bioactive metabolites with desired properties depends on the number and diversity of strain isolated and screened.[26]

- It has been clear that less than 1% of microorganisms in the natural environment can not be calculated using conventional culture technique, culture media, culture condition and detection of microbial colonies with naked eye.
- In the field of investigational search for new biologically active substances where more than 10,000 secondary metabolites of microbial origin have been discovered which are from actinomycetes.
- Actinomycetes are important microorganisms since more than 90% of practical antibiotics originate from them and two third of the biologically active substances of microbial origin are produced by them.[27]
- Actinomycetes are involved in the turnover of the organic matter xenobiotic compounds and are prolifolic source of antibiotics.[25]
- Filamentous soil bacteria belonging to genus streptomyces are widely recognized as industrially important microorganisms because of their ability to produce many kind of novel secondary metabolites.
- Different streptomyces species produce about 75% of commercially and medicinally useful antibiotics.[28]
- Streptomyces obtained from soil samples produced cytotoxic and antiviral antibiotics.[29]
- The resistance of numerous pathogenic bacteria presently an important area of antibiotic research. Natural products having novel structures have been observed to possesses useful biological activities.[1]

- Clavulanic acid is a naturally occurring antibiotic produced by streptomyces clavuligenus. The antibiotic itself exhibits only weak antibacterial activity against most bacterial, but it is a potent inhibitor of wide range of β- lactamase enzymes.
- The combined effective action of the β-lactamase inhibitor and antibacterial agent make clavulanic acid very important both clinically and economically.[30]
- Some atinomycetes are known to impart earthy odors and taste to reservoirs and supplies in paper mills.
- Actinomycetes are reported to produce abundant growth and thick foam activated sludge leading to the ineffective functioning of waste water treatment plants.
- The ability of streptomyces to degrade both the cellulose and lignin in lignicellulose has also been reported.[31]
- Actinomycetes are known to metabolise different phenyl propenoic acids.[32]

1.12 APPLICATIONS

- The actinomycetes are natural products that have been an extraordinary rich source for lead structures in the development of new successful drugs.
- The lead structures derived from actinomycetes were used as antimicrobials.
- The actinomycete produced repamycin, ascomycin acts as immunosuppresants.
- These were also acting as anticancer agents bleomycin, dactinomycin, doxorubin, which are derived from actinomycetes act as anticancer agents.

- These actinomycetes are also antifungal. The first isolated antifungal antibiotic Nystatin was produced from soil (*S.noursei*).
- The actinomycetes derived amphoterin B, also acts as antifungal agent.
- The actinomycetes are also acts as herbicides (Phosphinothricin).
- The actinomycetes play their role in the treatment of diabetes. The acarbose is used in the diabetes
- These are also used antihelmintic agents (avermectin, milbemycin).[33]
- The actinomycetes derived Myxoviromycin, kikumycin,and formycin acts as antiviral agent.[34]
- They are used in the identification of new drug targets by analysis of genomes of pathogens.[3]
- The actinomycetes are used in the biotransformation studies biotransformation of cinnamic acid and ferulic acid catalyzed by actinomycetes.[35]
- The actinomycetes produce various kinds of secondary metabolites.[36]
- They play their major role in production of various enzyme inhibitors.[37]

2. REVIEW OF LITERATURE

Literature review for Antiviral activity of Actinomycetes strain

1. **Sree Kumar et.al.;[12](2006)** carried out a study of aquaculture farms, particularly in Southeast asia are facing severe crisis due to increasing incidence of White Spot Syndrome Virus (WSSV). Actinomycetes have provided many important bioactive compounds of high prophylactic and therapeutic value and are continually being screened for few compounds. In this communication, results of a study made to determine the effectiveness of marine actinomycetes against the white spot disease in penaeid shrimps are presented. Twenty- five isolates of actinomycetes were tested for their ability to reduce infection due to WSSV among cultured shrimps. When these actinomycetes were made available as feed additives to the post- larvae of the black tiger shrimp *Penacus monoden* for two weeks challenged survival showed with WSSV, the post challenge survival showed variations from 11 to 83%.

2. **Yousry et.al.;[47](2006)** carried out a bioactive EPS producing microorganism, *Streptomyces nasri* was isolated from Kuwait tropical soil and the proteopolysaccharide was tested for its antimicrobial activity. The isolate was subjected to ultraviolet (UV) radiation and acridine orange (AO) treatment to select for superior proteo polysaccharide producers. Among eight (five derived from UV exposure and three from AO exposure) morphological variants of

Streotomyces nasri, two mutants showed increase production, from 1.8g/l to 2.3g/l.The polysaccharide was also tested for cytotoxic activity against human brain cell line using SRB assay.

3. **Sonya et.al.;[46](2005)** carried out four halotolerant *streptomyces* isolates (QSO1, QSO2, QSO3 **&** QSO4) were obtained from Quroon Lake, which had the ability to grow on 7% NaCl concentration in the starch agar medium. They were identified with a numerical method using two, three and four marker species belonging to red, grey and yellow series colour groups. Their antiviral activities against tobacco mosaic virus (TMV) and potato Y potyvirus (PVY) were also determined. The isolates QSO3 and QSO4 identified strains of *S.naganisii* and *S.michigansis* with similarities of 99.0 and 92.3%, respectively. QSO1 and QSO2 isolates were identified as duplicate strains of *S.erythracus* with similarities of 93.8 and 90.2% respectively. The numerical system was found to be a useful method for identification of the streptomycete isolate and could be limit the nomenclature of the new species of streptomycetes as well as the related genera of actinomycetes. As interesting, both of filtrates and pellets (Cells of streptomycetes) were found to contain substances with antiviral activities, as the number of necrotic local lesions (NLL) produced by TMV and PVY on *Nicotiana glutinosa* and *Chenopodium quinoa* respectively were decreased.

4. **Angelova et.al.;[13](2005)** carried out an extracellular proteinase inhibitor with alkaline phosphatase activity was produced by *Streptomycis chromofocus* .

Production of the proteinase was closely associated with mycelial growth. The origin of carbon source did not significant effect the production of inhibitor. The specific uptake of starch and glucose ($gl^{-1}h^{-1}$) are similar in the both cases of carbon sources. The specific proteinase inhibitory production rate was high in the presence of glucose (about 33%). The production of alkaline phosphatase showed two phase kinetics. During the early exponential phase the alkaline phosphatase activity has a maximum at 24 h of cultivation. The next maximum was found to be at the end of cultivation. The highest specific alkaline phosphatase activity was determined at the second maximum during the cultivation of strain (3800 mU /mg). Specific alkaline phosphatase production rate U l^{-1} h^{-1}) is higher in the presence of glucose. The proteinase inhibitor produced by *Streptomyces chromofuscus* showed significant protective effect in the murine model of experimental influenza virus infection.

5. **Carlo Maullu et.al.;[11](1998)** carried out a new method for screening microbial colonies endowed with antiviral activity is described. It is based on close contact between microbial agar cultures and agar-covered virus- infected-cell monolayers and allows the screening of large numbers of colonies in just a few months.

6. **Nzunzu Lami et.al.;[48] (1993)** carried out a microbial diversity is a key element in the search of useful new secondary metabolites. In this aim, we screened microorganism strains (actinomycetes and fungi) isolated from Zairian (Africa)

soil samples for antimicrobial, antiviral, and antitumor activities. About one third of these strains exhibited biological activities with remarkable prevalence of antitumor as well as antiviral activities.

7. **Nobuaki Naurse et.al.;[43](1989)** carried out a new antibiotic Pumilacidins A,B,C,D,E,F and G were isolated from the culture broth of a strain of *Bacillus pumilus.* They are cyclic acylheptapeptide composed of a ß-hydroxy fattyacid, two L-leucine, two D-leucine, L-glutamic acid, L-aspartic acid and L-isoleucin (or L-valine). Pumilacidin components were inhibitory to herpes simplex virus type I.

8. **Hiroyoshi Tohyama et. al.;[42](1984)** carried out a new Indole N glycoside antibiotic, SF-2140 which shows antiviral weak anti bacterial activity has been obtained from the cultured broth of an actinomycetes strain. Strain SF-2140 designated *Actinomadura alolutea* sp, was isolated from a soil simple collected in Hyogo Prefecture, Japan.

9. **Noriyuki Satto et.al.;[34](1973)** studied a new antiviral agent which inhibits the growth of polio virus, vesicular stomatitis virus (VSV**)** and Newcastle disease virus (NDV) was isolated from the culture filtrate of a *Streptomysis* and shown to be 3-(5,7- dimethyl-4- oxo-2- hydroxy-6, 8-decadienyl)-glutrimide. The acute LD50 of the antibiotic in mice by intraperitoneal injection was 200 mg/kg to mice inhibited the growth of polio virus.

10. **Kei arima et.al.;[45](1972)** studied Tomaymycin, a new antibiotic is produced by cultivation of *streptomyces achromogenes* , *tomaymyceticus* in a lactose-bouillon medium enriched with yeast extract and phosphate salts. The active substance can be extracted from the culture filtrate by carbon adsorption and further purified by silica column chromatography. Tomaymycin is obtained in crystalline form as a molecular complex with methanol. The complex occurs as colorless platelets, m.p. 145~146°C (0.5, pyridine), y max 224, 237, 260, 320 mμ (in methanol). The molecular formula is $C_{16}H_{20}O_4N_2$. The antiphage activity is strong against *Escherichia coli* T1, T3 phages and *Bacillus subtiles* M-2, SP-10 phages.

11. **Kri Arima et. al.;[29](1972)** carried out the screening for antiviral antibiotics produced by *Streptomyces* soil isolates, several strains were found to produce cytotoxic and antiviral antibiotics. One antiviral designated S-15-1 was isolated from soil isolate *Streptomyeis*S-15-1.The antibiotic S-15-1 shows noncytotoxic *invitro* inhibitory activity against Newcastle virus, and it has also inhibitory activity against gram-positive, gram-negative bacteria as well as some yeasts.

12. **Kunio et.al,;[44](1968)** studied Mycophenolic acid shows significant antiviral activity in the agar diffusion, plaque inhibition and tube culture methods. Both DNA and RNA viruses are involved in the antiviral spectrum and the chemotherapeutic index of Mycophenolic acid is high against *invitro*.

Literature survey for DNA isolation from Actinomycetes strains.

1. **Abate et.al.;[24](2005)** carried out isolation and characterization of indigenous copper-resistant actinomycete strains, in their work they isolated and purified the actinomycetes DNA, from strains grown in liquid for 4 days.The pellets was collected by centrifugation and washed sterile distilled water.and total genomic DNA extraction was carried out for ABO strains.

2. **Stefano et.al.;[3](2002)** studied microbial technologies, for the discovery of novel bioactive metabolites, their study review the development and application of molecular methods for the detection of un common genera of actinomycetes in soil DNA. The results indicate that to speed of stability, maintaining large segments of actinomycetes DNA in *Escherichia coli* and of integrating site specifically in the streptomyces genome.

3. **Macneil et.al;[58] (2001)** carried out the screening of the library resulted in the identification of several antimicrobial activities expressed by different recombinant clones. One clone (mg1.1) has been partially characterized and found to express several small molecules related and including indirubin. These results show that genes involved in natural product synthesis can be cloned directly from S-DNA and expressed in a heterologous host.

4. **Harry et.al.;[39](2001)** carried out the extraction and purification of microbial DNA from soil, and sediment sample. They followed and for purification of DNA studied two methods direct lysis and cell lysis methods for extraction of DNA. And for purification their protocol includes CsCl ultracentrifugation, chromatography, electrophoresis, dialysis and filtration results shows that they extracted and purified DNA form soil samples by cell lysis method.

5. **Eric Triplet et.al.;[49](1996)** studied molecular microbial diversity of an Agricultural soil in wisconsin. In their study a culture –independent survey of the soil microbial diversity in a clover-grass pasture in southern wisconsin was conducted by sequence analysis of a universal clone library of gene coding for small subunit (rDNA). A rapid and efficient method fur extraction of DNA from soils which resulted in highly purified DNA with minimal shearing was developed.

6. **Erko stackebrandt et.al.;[50](1996)** carried out a study of the bacterial flora associated with *Holothuris atra*, a study was undertaken to investigate the aerobic bacterial flora of the seacucumber *Holothuria utra* and the bacterial population of the animals immediate environment and food source (marine sediment). They extracted and purified microbial DNA from gut bacteria studied for rDNA analysis. Results indicates that from isolation study, 43 strains was selected for identification and characterization by 16s ribosomal DNA sequence analysis and limited phenotypic testing.

3. AIM AND OBJECTIVES

The search for antibiotics continues to be of extreme importance in research programs around the world because of the increase of resistant pathogens and toxicity of some used antibiotics. The history of new drug discovery possesses shows that novel skeleton have, in the majority of cases, come from natural sources. This involves the screening of microorganisms and plant extracts. Among the microorganisms, actinomycetes are one of the most attractive sources of antibiotics and other biologically active substances of high commercial value and from which, *Streptomyces* species has been the most fruitful source of all types of bioactive metabolites that have important applications in human medicine as antiviral and anticancer compounds. Thus, screening and isolation promising strains of actinomycetes with potential antibiotics still a thrust area of search for many years.

As all we know that the major causative agent for many diseases among major populations all over the world. To eradicate some type of virus completely by using cheap available source like actinomycetes this study was aimed.

So in this present study the aim is to isolate actinomycete strain from the soil sediments which is having antiviral activity and analysis for Thin layer chromatography, UV, F.T.I.R, Mass spectroscopy.

4. SCOPE AND PLAN OF WORK

Streptomycetes are ubiquitous gram positive soil bacteria belonging to the order Actinomycetales. Fewer and fewer new drugs have been found in largest screening programs during the past decades, and scientists have started working for the new technologies to generate new compounds. Approximately two-thirds of the known microbial secondary metabolites are produced by the members of the order Actinomycetales.

Enormous effort is being made worldwide by microbial ecologist to identify microorganisms in environmental samples. Based on the above, facts my plan of work was framed as

1. Collection of soil samples from different region of Tamilnadu.
1. Isolation of actinomycetes colonies from soils.
    a. Isolation of Actinomycetes by Glucose yeast extract malt agar medium.
    b. Isolation of Actinomycetes by Luria- Bertani agar medium.
    c. Isolation of Actinomycetes by Inorganic salt agar medium.
2. Purification of selected actinomycetes strains.
3. Cultural characteristics of selected actinomycetes strains.
4. Morphological Characteristics of selected actinomycetes strain.
5. Bio-chemical characteristics of selected actinomycete strain.
6. Production of active compounds by shake flask method.

a) Fermentation by Glucose yeast extract malt broth.

b) Fermentation by Luria-Bertani broth.

c) Fermentation by Inorganic salt broth.

7. Recovery of the fermentation product.
8. Extraction of active compound from supernatant by ethyl acetate, actone and methanol.
9. Antiviral activity.
10. Extraction of microbial DNA of selected actinomycete strain.
11. Purification of microbial DNA of selected actinomycete strain.
12. Detection of DNA by Gel- documentation system.
13. Analysis of the compounds by

a) TLC

b) UV spectroscopy.

c) F.T.I.R spectroscopy

d) Mass spectroscopy.

5. PROFILE

SOIL:

Soil has been defined as that region on the earth`s crust where geology and biology meet. From the functional view point, the soil may be considered as the land surface of the earth which provides the substratum for plant and animal life. The characteristics of the soil environment vary with local and climate. Soil differs in depth, physical properties, chemical composition and origin.

Soil Profile **Table-1**

Soil profile of samples used for study

Sr.No	Soil code	Collection from	Texture	Colour	pH
1.	S_1	Coimbatore Dt	Hard	Red	6.8
2.	S_2	Coimbatore Dt	Hard	Brown	5.8
3,	S_3	Coimbatore Dt	Hard	Brown	6.3
4.	S_4	Coimbatore Dt	Hard	Red	7.1
5.	S_5	Coimbatore Dt	Smooth	Black	7.6
6.	S_6	Coimbatore Dt	Smooth	Brown	8.1
7.	S_7	Coimbature Dt	Smooth	Red	7.3
8.	S_8	Erode Dt	Smooth	Brown	8.2
9.	S_9	Erode Dt	Smooth	Grey	8.5
10.	S_{10}	Erode Dt	Smooth	Black	5.8
11.	S_{11}	Erode Dt	Smooth	Black	6.7
12.	S_{12}	Erode Dt	Hard	Grey	8.3
13.	S_{13}	Erode Dt	Hard	Brown	7.7
14.	S_{14}	Erode Dt	Hard	Brown	7.4
15.	S_{15}	Salem Dt	Hard	Red	6.7
16.	S_{16}	Salem Dt	Hard	Red	6.9
17.	S_{17}	Salem Dt	Smooth	Brown	7.2
18.	S_{18}	Salem Dt	Smooth	Brown	7.4
19.	S_{19}	Salem Dt	Smooth	Black	5.7
20.	S_{20}	Salem Dt	Smooth	Brown	7.6
21.	S_{21}	Nagai Dt	Smooth	Red	7.5
22.	S_{22}	Nagai Dt	Smooth	Brown	6.8
23.	S_{23}	Nagai Dt	Hard	Brown	8.5
24.	S_{24}	Nagai Dt	Hard	Red	7.4
25.	S_{25}	Nagai Dt	Hard	Brown	8.3

6. METHODOLOGY

1. Collection of Soil Samples

- 25 samples were collected from the region of Coimbatore, Erode, Salem, Nagai Districts of Tamilnadu in a sterile plastic bags from the depth of 5to 15 cm.
- Soil samples were air dried and stored at 4°C until further examination.
- Mean while pH and texture of soil samples can be tested. [1,25].

2. Surface Sterilization of Soil

From the collected soil samples, weighed accurately about 1gm, and, to that 5ml of Phenol was added, which acts as surface acting agent, now it was diluted with water made upto and shake vigorously for one hour.

From that, serial dilution was made by taking 1ml from this solution and it was added to the test tube and dilution was made from 10^{-1} to 10^{-10}dilution.[59]

3. Isolation of Actinomycetes colonies from soils

Isolation and Enumeration of Actinomycetes was performed by ten fold serial soil dilution technique.

a) Isolation of Actinomycetes by Glucose – Yeast Extract Malt Agar medium

- 0.2ml of each dilutions (10^{-5}, 10^{-7}, 10^{-9}) were placed on Glucose-Yeast Extract Malt Agar medium by spread plate technique.
- The plates were prepared in triplicates.
- The plates were incubated at 27°C for 2 weeks.
- The growth of Actinomycetes were observed after 7days.[17]

b) Isolation of Actinomycetes by Luria-Bertani Agar medium.

- 0.2ml of each dilutions (10^{-5}, 10^{-7}, 10^{-9}) were placed on Luria-Bertani agar medium by spread plate technique.
- The plates were prepared in triplicates.
- The plates were incubated at 27°C for 2 weeks.
- The growth of Actinomycetes were observed after 7days.[18]

c) Isolation of Actinomycetes by Inorganic Salt Agar medium

- 0.2ml of each dilutions (10^{-5}, 10^{-7}, 10^{-9}) were placed on Inorganic Salt Agar medium by spread plate technique.
- The plates were prepared in triplicates.
- The plates were incubated at 27°C for 2 weeks.
- The growth of Actinomycetes were observed after 7days.[3]

4. Purification of Actinomycetes

- Selected colonies of actinomycetes from Glucose-Yeast Extract Malt Agar medium, Luria-Bertani Agar medium, and Inorganic Salt agar Medium were transferred into these respective medium.
- The plates were incubated at 27°C for 2 weeks.
- The slant cultures of respective medium were prepared and stored at 4°C until further examination.[1]

5. Cultural Characteristics [2]

The cultural characteristics of isolated actinomycetes were studied in which colour of a aerial mycelia, colour of substrate mycelia, and fragmentation of the isolates were observed.[26]

6. Morphological Characteristics

The morphological characteristics of selected actinomycetes strain were studied by

a) Gram staining

b) Motility staining

c) Acid Fast staining

d) Spore staining.[51]

7. **Bio-chemical characteristics** [51,52,22]

a) Test for melanoid formation

- The isolate was streaked on the slant of Waksman medium and incubated at 28ºC for 4 days.
- The slant was observed for melanoid formation for every 12 hrs.

b) Test for nitrate reduction

- To the sterilized organic nitrate broth a loopful isolate was transferred and incubated at 28°C.
- From the fifth day the tubes was observed for nitrate reduction.
- The reagents used
  a) α- napthalein solution
  b) Sulphonic acid solution.
- To 1 ml of the broth under examination 2 drops of reagent (a) and 2 drops of reagent (b) was added.
- A positive reaction shows pink colour the observation was recorded.

c) Test for hydrogen sulphide

- The isolates was streaked on medium for hydrogen sulphide and incubated for 24 days at 28°C.
- The observation greenish brown, bluish black at every 12 hrs for 4 days.
- Observation was done.

e) Test for acid production

- The test organism was inoculated in to glucose nutrient broth and incubated at 25°C for 4 days.
- At every 12 hrs interval any change in colour noted blue to yellow indicate acid production.
- Bromophenol blue was used as indicator.

8. Production of active compound by shake flask method [26,53]

- For the production of active compounds the Glucose-Yeast Extract Malt Agar medium, Luria-Bertani Agar medium, and Inorganic Salt agar Medium.

a) Fermentation by Glucose –Yeast extract Malt broth.[57]

- The composition of broth medium were accurately weighed and dissolved in 100ml of distilled water and sterilized at 121°C (15 lbs) for 15 minutes by using autoclave and allow to cool.
- 10% culture of two strains of AcO2, AcO6, were inoculated in to Glucose-Yeast extract Malt broth and kept for fermentation on rotary shaker for 14 days at 200 rpm at 30°C.

b) Fermentation by Luria-Bertani broth[55]

- The composition of broth medium were accurately weighed and dissolved in 100ml of distilled water and sterilized at 121°C (15 lbs) for 15 minutes by using autoclave and allow to cool.
- 10% culture of two strains of AcO10, AcO15 were inoculated in to Luria-Bertani broth and kept for fermentation on rotary shaker for 14 days at 200 rpm at 30°C.

c) Fermentation by Inorganic Salt broth [54]

- The composition of broth medium were accurately weighed and dissolved in 100ml of distilled water and sterilized at 121°C (15 lbs) for 15 minutes by using autoclave and allow to cool.
- 10% culture of two strains of AcO20, AcO23 were inoculated in to Inorganic salt broth and kept for fermentation on rotary shaker for 14 days at 200 rpm at 30ºC.

9) Recovery of the fermentation product[26]

Broth was taken at the end of the 14th day and it centrifuged at 10,000 rpm, temperature at 4ºC for 10 minutes to separate the mycelial biomass, the supernatant were obtained, and it was separated by filtration using Whatmann filter paper.

10) **Extraction of active compound**[54]

The supernatant of S_2, S_6, S_{10}, S_{15}, S_{20}, S_{23} was mixed with the equal volume of solvents were ethyl acetate, acetone and methanol. The solvent supernatant mixture was agitated for 45 mts, with homogenizer. The solvent was separated from broth by separating funnel, solvent present in the broth was separated by centrifugation at 5000 rpm for 15 mts, to remove traces of fermentive broth. The extracts were evaporated by water bath at 87°C until the dark brown gummy substance was obtained. The crude antibiotic was collected and dried in oven at 40°C. Residue obtained was subjected to purification.

12) Antiviral activity of the compounds

Virus and cells.

The strain of HSV-1 was propagated in Vero cells. All cells were grown in MEM with 10% fetal bovine Serum.

Antiviral assays.

The antiviral activity of extracts was assayed in 96-well microtiter plates wiyh 30,000 cells/well. The cell culture was incubated at 37ºC for 24 hrs in a humidified 5% CO_2 atmosphere. After 24 h of incubation, add 100 µg and 50µg of extracts at dilutions. The cells were incubated for 1h. After this period, 50µl of logarithmic dilutions of viruses were added in triplicate. The plates were incubated again for 96 h. Monolayers of cells

incubated only with MEM were used as a control. Viral titers were determined by 50% infective doses in tissue culture-TCID50 (Reed & Muench 1938). Antiviral activities were calculated as the difference of virus titer between treated and untreated infected control cultures.[59]

11 Extraction of microbial DNA from Actinomycete strains [39,54]

1. 2ml of broth was taken and centrifuged at 12000 rpm at room temperature, pellet was collected.
2. Suspended the pellet in to 2ml of NaCl Buffer and 250µl of SDS mixed well and incubated of 60°C for 10min.
3. To above mixture added phenol : chloroform (1:2) mixture mixed well and centrifuged 12000 rpm of 4°C for 10 minutes, collected the supernatant.
4. To the supernatant added ice cold isoproponol twice the volume incubated for 30 minutes.
5. Spin it at 12000rpm for 10 min at 4°C and suspended the pellet in 300µl of TE buffer.

Purification of DNA[54]

a) Sealed the edges of a clean, dry glass plate (or the open ends of the plastic tray supplied with the electrophoresis apparatus) with tape to form a mold set the mold on a horizontal section of the bench.

b) Prepared sufficient electrophoresis buffer (usually 0.5× TBE) to the electrophoresis tank and to cast the gel.

c) Prepared a solution of agarose in electrophoresis buffer at a concentration appropriate for separating the particular size fragments expected in the DNA sample. Added correct amount of powdered agarose (0.8%) to a measured quantity of electrophoresis buffer in an Erlenmeyer flask or a glass bottle.

d) Loosely plug the neck of the Erlenmeyer flask with kimpipes. Heat the slurry on a boiling water bath until the agarose dissolved.

e) Used insulated gloves to transfer the flask in to a water bath at 55°C when the molten gel has cooled, add a ethidium bromide to final concentration of 0.5mg/ml, mixed the gel solution thoroughly by gentle swirling.

f) While the agarose solution was cooling choose an appropriate comb for forming the appropriate slots in the gel, position the comb 0.5- 1mm above the plate so that complete well was formed when agarose was added to the mold.

g) Poured the warm agarose solution in to the mold.

h) Allowed the gel to set completely (30-45 minutes at room temperature), then pour in to a small amount of electrophoresis buffer on top of the gel, and carefully removed tape mounted the gel in the electrophoresis tank.

i) Added just enough electrophoresis buffers to cover the gel to a depth of 1mm.

j) Mixed the samples of DNA with 0.20 volume of the desired 5× gel loading buffer.

k) Slowly load the sample mixture in to the slot of the submerged gel using a disposable micropipette.

l) Loaded the size standard into first and last slot of the gel.

m) Close the lid of the gel tank and attached the electrical leads, so that the DNA will migrate toward the positive anode (red lead). Apply a voltage of 1-5 V/cm (measured as the distance between the positive and negative electrodes). If the leads have been attached correctly, bubbles should be generated at the anode and cathode (due to electrolysis) and within few minutes the bromophenol blue should migrate from the wells in to the body of the gel. Run the gel until the bromophenol blue have migrated an appropriate distance, through the gel.

n) When the DNA samples or dyes have migrated a sufficient distance through the gel, turned off the electric current and removed the leads and lid from the gel tank if ethidium bromide is present in the gel and electrophoresis buffer, examine the gel by UV light.

3. Detection of DNA[54]

In order to detect the DNA which was separated by gel electrophoresis the gel was placed on the gel documentation system under UV- Transilluminator and the bands were observed.

13) Analysis of the compounds[28]

a) Thin layer Chromatography

- 5mg of the resulting dry extracts of AcO2 were recaptured in 5ml of dimethyl sulfoxide (DMSO)
- Analyzed by thin layer chromatography (TLC) using silicagel (1:2)
- The organic solvents were 5% chloroform and 95% methanol.
- The TLC was run for AcO2 extract and visualized with ultraviolet light and iodine vapors.
- The Retention factor (Rf) were calculated for the extracts.
- The number of active compounds separated were observed.

b) UV Spectroscopy

- The λmax of the compound AcO2 was deter mined by UV spectroscopy
- For the compound AcO2, 0.1 Hcl was used as solvent.
- The λmax was recorded for the AcO2.

b) FTIR Spectroscopy

- The infra red spectrum of the force extract i.e AcO2 was recorded.
- The KBr pellets were prepared by pressed pellet technique and these pellets were used for FTIR spectra.

c) Mass spectroscopy

The mass spectrum of antiviral compound was taken using EIMS at 328 scan and graph was plotted as m/z Vs base peak.

7. MATERIALS AND INSTRUMENTS

MATERIALS

S.NO	NAME	COMPANY
1.	Agarose	HIMEDIA, Mumbai.
2.	Acivir	Cipla PVT., Goa.
3.	α - Napthalein	HIMEDIA, Mumbai.
4.	Beef extract	HIMEDIA, Mumbai.
5.	Bromo phenol blue	HIMEDIA, Mumbai.
6.	Crystal violet	HIMEDIA, Mumbai.
7.	Carbol fuschin	HIMEDIA, Mumbai.
8.	Di Potassium hydrogen phosphate	HIMEDIA, Mumbai.
9.	Distilled water	Leo scientific, Erode.
10.	EDTA	LOBA PVT., Mumbai.
11.	Ethidium Bromide	HIMEDIA, Mumbai.
12.	Ferric ammonium sulphate	LOBA PVT., Mumbai.
13.	Ferrous sulphate	LOBA PVT., Mumbai.
14.	Glucose	LOBA PVT., Mumbai.
15.	Grams Iodine	HIMEDIA, Mumbai.
16.	Gelatin	HIMEDIA, Mumbai.
17.	L- Tyrosine	HIMEDIA, Mumbai.
18.	Magnesium Sulphate	LOBA PVT., Mumbai.
19.	Malt Extract	HIMEDIA, Mumbai.
20.	Marker DNA	Genei, Bangalore.
21	Meat Extract	HIMEDIA, Mumbai.
22.	Muller -- Hinton Agar	HIMEDIA, Mumbai.
23.	Nystatin	HIMEDIA, Mumbai.
24.	Peptone	HIMEDIA, Mumbai.
25.	Potassium Di Hydrogen Phosphate	LOBA PVT, LTD., Mumbai.
26.	Potassium Nitrate	LOBA PVT, LTD., Mumbai.

27.	Sodium propionate	LOBA PVT, LTD., Mumbai.
28.	Sodium Chloride	HIMEDIA, Mumbai.
29.	Sodium thio sulphate	Sd.Fine chemical LTD, Mumbai.
30.	Saffranine	HIMEDIA, Mumbai.
31.	Sulphonic Acid	LOBA PVT, LTD., Mumbai.
32.	Sucrose	Nice chemical, LTD., Cochin.
33.	Tris HCL	HIMEDIA, Mumbai.
34.	Tris Base	HIMEDIA, Mumbai.
35.	Yeast Extract	HIMEDIA, Mumbai.

Solvents used

1. Methanol
2. Ethyl acetate
3. Chloroform
4. Acetone
5. n- butanol
6. Dimethyl sulphoxide (DMSO)

All solvents used were of analytical grade.

ORGANISM USED

Herpes simplex virus type I

INSTRUMENTS

S.NO	NAME	COMPANY
1.	Cold centrifuge	REMI
2.	Electric water bath	GENUINE
3.	Deep freezer	BLUE STAR
4.	Research centrifuge	REMI
5.	Rotary shaker	GENUINE
6.	Hot air oven	GENUINE
7.	Refrigerator	GODREJ
8.	Laminar air flow(verticle)	GENUINE
9.	Incubator	GENUINE
10.	Gel documentation system UST-15-8K	BIO step
11.	Analytical balance	SHIMADZU, Japan
12.	IR- Spectrometer	SHIMADZU, Japan
13.	Optical microscope US-33-RP	DOLLER
14.	PH-meter (Digital)	ELICO
15.	Vacuum filter	SHREEJI
16.	Autoclave	NEW LAB
17.	Horizontal Gel Electrophoresis	YERCAUD BIO-TECH
18.	EIMS	SHIMADZU, Japan
19	UV spectrophotometer	ELICO

8. RESULTS AND DISCUSSION

1) Isolation of actinomycetes

6 Actinomycetes strains were recovered from the soil samples collected from the Coimbatore, Erode, Salem and Nagai districts regions of Tamilnadu by using Glucose- yeast extract malt agar medium, Luria – bertani agar medium and Inorganic salt agar medium. From the 6 isolates the 2 actinomycetes strains were recovered from Glucose- yeast extract malt agar medium,2 actinomycetes strains were recovered from Luria – bertani agar medium and 2 actinomycetes strains were recovered from Inorganic salt agar medium.

2) Purification of actinomycetes

6 Actinomycetes strains were purified by transfer of strains to their respective medium after incubation of 7 days the pure actinomycetes strains recovered (Ac01 to Ac06).

3) Cultural characteristics

The cultural characteristics of the actinomycetes strains Ac01 to Ac06 were studied in which the colour of the aerial mycelia observed as yellowish, pink, white, light grey for some actinomycetes strains and the colour of substrate mycelia also varied for each actinomycetes sp. The fragmentation was not shown by most of the actinomycetes strains except few one with little fragmentation.

The cultural characteristics of isolates Ac01 to Ac06 was given in table no.2

4) Morphological Characteristics

The morphological characteristics of the isolates actinomycete strains Ac01 to AcO6 was studied by gram staining, motility staining , acid fast staining, and spore staining. The strain Ac01, AcO2, AcO4 and AcO6 were the gram positive filamentous bacteria, AcO3 and AcO5 were the gram filamentous bacteria. AcO1, AcO3, and AcO6 were motile, AcO2, AcO4 and AcO6 were non motile, AcO1 to AcO6 were non acid fast with few spores. The morphological characteristics was indicated as compared with Bergey`s manual volume 4 that the strain Ac02, AcO4 was related to streptomyces sp.

The morphological characteristics were summarized in table no. 3

5) Biochemical characteristics

The various biochemical characteristics were studied for the actinomycetes strains Ac01 to AcO6. The Ac01 to AcO6 strains were not shows melanoid production. It shows negative response for gelatin liquefication, nitrate reduction and production of hydrogen sulphide. The acid was produced by the strain Ac01 to AcO6 as they shows yellow colour when bromophenol blue was used as indicator. The biochemical characteristics were summarized in table no.4

6) Production and extraction of active compounds

The Ac01 to AcO6 strains were used for the fermentation by using Glucose-yeast extract malt broth, Luria – bertani broth and Inorganic salt broth. The fermentation process was completed at 30°C in 14 days. After fermentation process, the supernatants were obtained by filtration and centrifugation. The three extracts was obtained by evaporation technique of solvents, out of which AcO1, AcO3 and AcO5 were brown colour, AcO2, AcO4 and AcO6 yellow powder. The weights of extracted powders (AcO1 to AcO6) were found to be 1.12g, 1.24g, 1.32g, 1.26g, 1.28g and 1.37g respectively.

7) Antiviral activity

Results suggest that the methanol and acetone extracts showed antiviral activity against Herpes simplex virus type I. Results are shown in table no.6

8) Extraction of DNA

The DNA of actinomycetes strain Ac02 was isolated by cell lysis method the DNA bands were purified by Agarose gel electrophoresis. The bands of DNA were detected by using UV Transilluminator in gel documentation system.

The bands of DNA of Ac02 strain were separated according to their molecular weight and are compared with marker DNA. The DNA obtained was free of contaminants like humic acid, but few contaminants of phenolic substances were present.

The results of Agarose gel electrophoresis were shown in Fig No.6

9) Analytical Methods

The three extracts were analyzed by thin layer chromatography the one active compounds were shown by AcO2 and the Rf value of methanol and acetone extract of AcO2 were found as 0.86 and 0.87.

The TLC plates of AcO2 was shown in fig no.7

By using Elico 24 double beam UV spectrometer the λmax of the AcO2 was found to be 232nm respectively.

The λmax plots of AcO2 was given in graph no.1 and table no.6

The IR spectrum of the AcO2 was recorded. The functional group present in AcO2 was C-H aromatic bending (3211.86 cm^{-1}), C=N (2350cm^{-1}), C=0 (1594 cm^{-1}) , C=C (1395 cm^{-1})The IR spectrum of the was given in Graph No.1.

The mass spectrum of the AcO2 was recorded. The mass spectrum of the AcO2 was given in Graph No.2

Table 2: Cultural characteristics of the actinomycetes strains.

S. No	Isolation Medium	Soil used	Isolate code	Colour of Aerialmycelia	Colour of Substrate mycelia	Fragmentation
1.	GLM	S2	Ac01	White	White	None
2.	GLM	S6	Ac02	Brownish	Yellow	None
3.	LB	S10	Ac03	Yellow	Brown	Very little
4.	LB	S15	Ac04	White	Yellow	None
5.	ISP	S20	Ac05	Yellow	Brown	None
6.	ISP	S23	Ac06	Brown	Yellow	None

Table 3

MORPHOLOGICAL CHARACTERISTICS OF ACTINOMYCETE STRAINS

S.No	Test	AcO1	AcO2	AcO3	AcO4	AcO5	AcO6
1.	Gram staining	Gram positive	Gram positive	Gram negative	Gram positive	Gram negative	Gram positive
2.	Motility	Motile	Non motile	Motile	Non motile	Non motile	Motile
3.	Spore staining	Few spores	Few spores	Few spores	Few spores	Few spores	Few spores
4,	Acid fast staining	Non acid fast	Non acid fast	Non acid fast	Non acid fast	Non acid fast	Non acid fast

Table 4

BIOCHEMICAL CHARACTERISTICS OF ACTINOMYCETE STRAINS

S.NO	TEST	AcO1	AcO2	AcO3	AcO4	AcO5	AcO6
1	Melanoid formation	-	-	-	-	-	-
2	Nitrate reduction	-	-	-	-	-	-
3	Hydrogen sulphide	-	-	-	-	-	-
4	Gelatin liquefaction	-	-	-	-	-	-
5	Acid production	+	+	+	+	+	+

Table no-5

Antiviral activity of AcO2 against Herpes simplex virus type-1

S.NO	EXTRACTS	Concentration tested (µg/ml) IC50	CPE INHIBITION ASSAY		
			2TCID50	10TCID50	100TCID50
1	Methanol	100	++++	+++	++++
		50	+++	++	++
2	Acetone	100	++++	++++	+++
		50	++	++	+

+ 25% protection

++ 50% protection

+++ 75% protection

++++100% protection

CPE: cytopathic effect

IC 50: Inhibititory concentration

TCID: Tissue culture infective dose

UV Spectroscopy of AcO2 strain

Table no.6

S.no	Wave length in nm	Absorbance
1	220	0.276
2	222	0.278
3	224	0.282
4	226	0.285
5	228	0.291
6	230	0.294
7	232	0.296
8	234	0.293
9	236	0.290
10	238	0.288
11	240	0.282
12	242	0.278
13	244	0.274
14	246	0.286
15	248	0.260
16	250	0.254

Graph -1
UV Spectroscopy of AcO2 strain

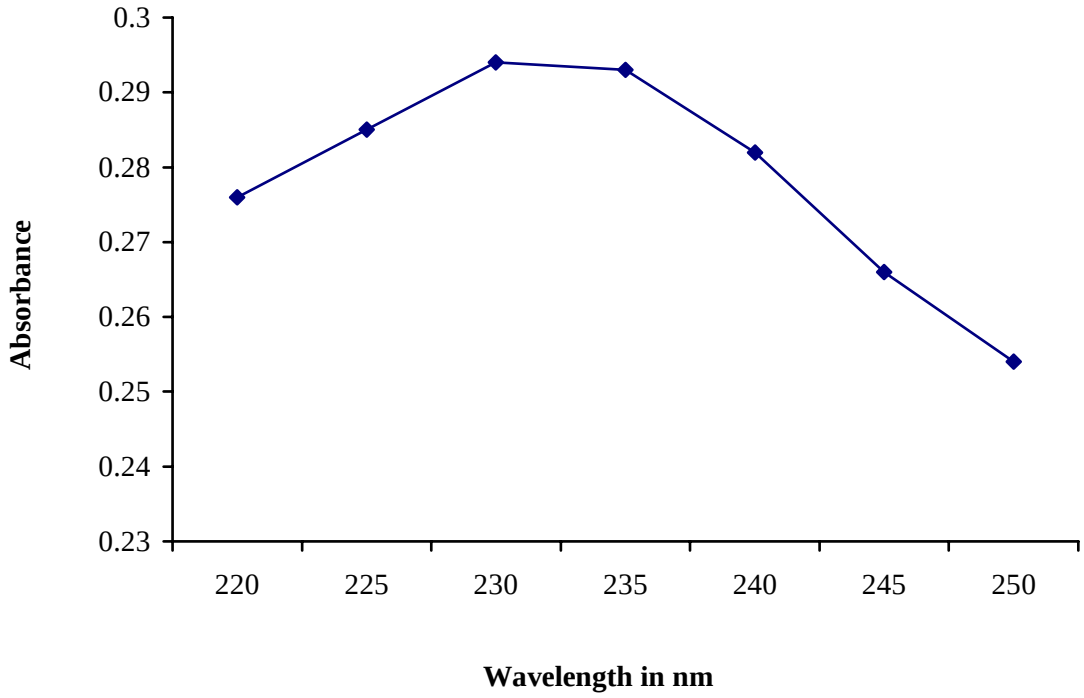

Figure no-1

Mono layers of cell incubated only with MEM as a control

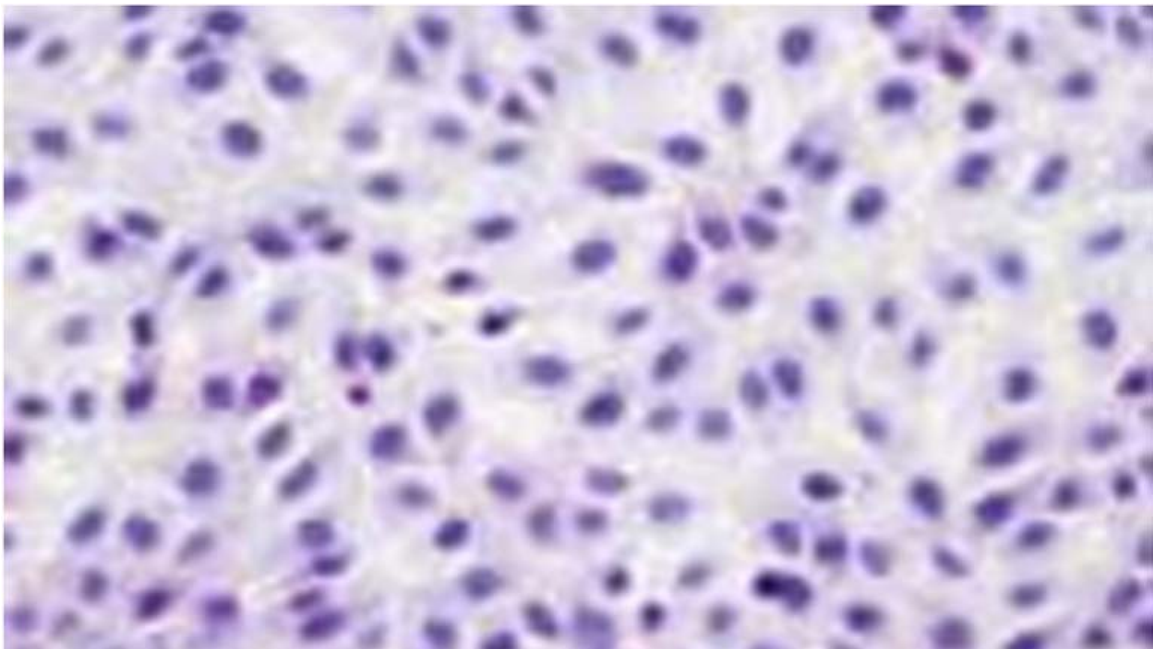

Figure no-2

Antiviral activity of methanol extract of AcO2 (50µg) against Herpes simplex virus type-2

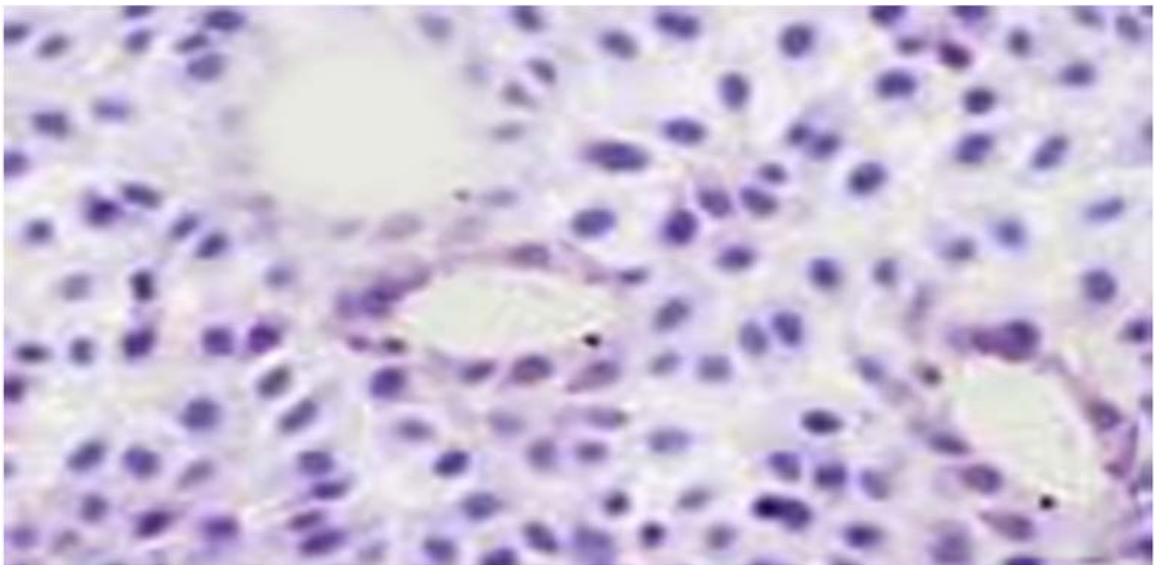

Figure no-3

Antiviral activity of methanol extract of AcO2 (100µg) against Herpes simplex virus type-2

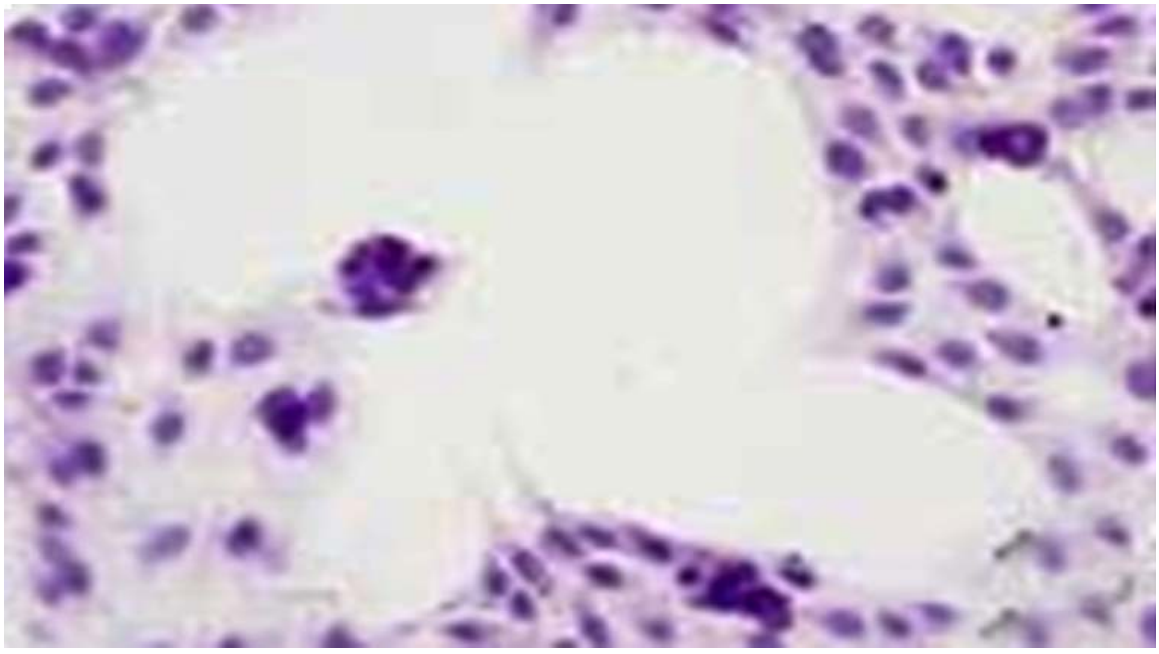

Figure no-4
Antiviral activity of acetone extract of AcO2 (50µg) against Herpes simplex virus type-2

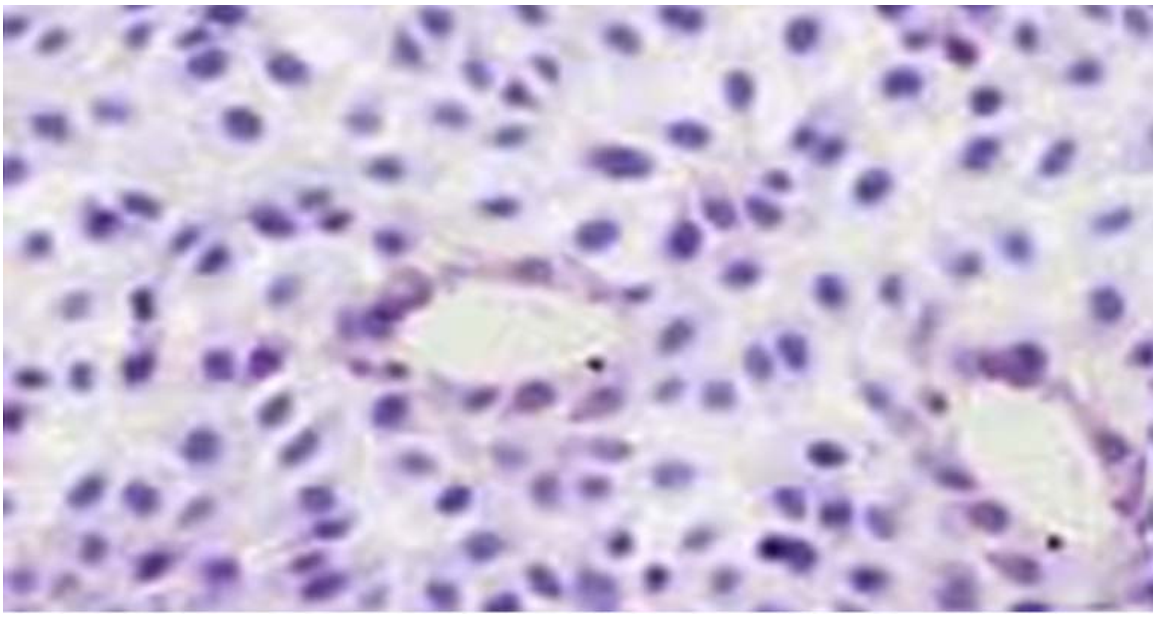

Figure no-5
Antiviral activity of acetone extract of AcO2 (100µg) against Herpes simplex virus type-2

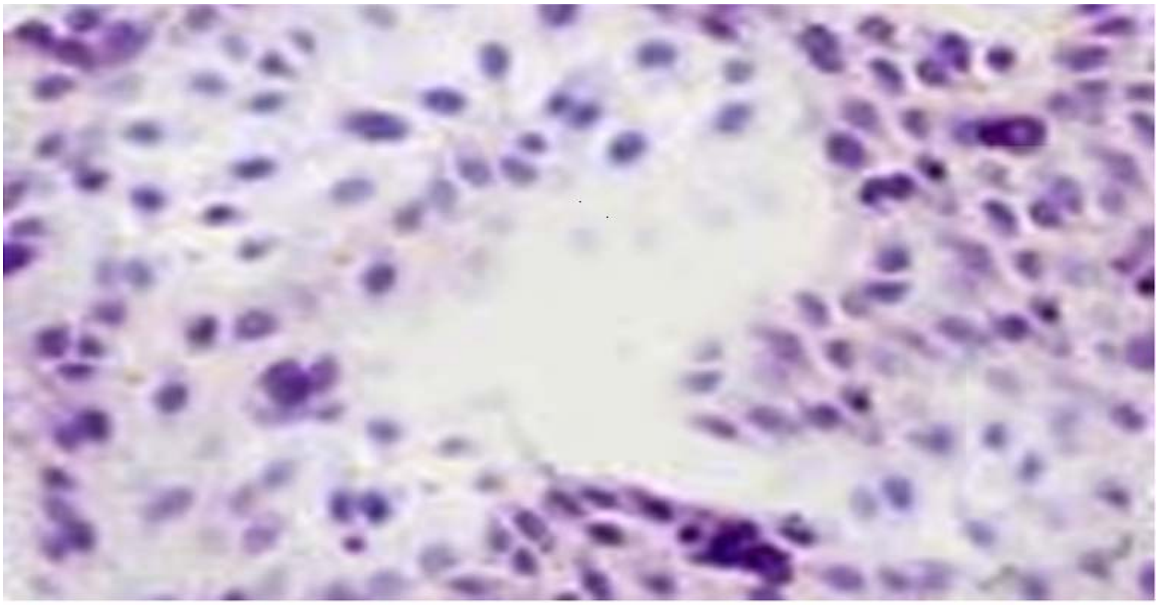

Figure no-6
The DNA bands of AcO2 strain by Gel Electrophoresis.

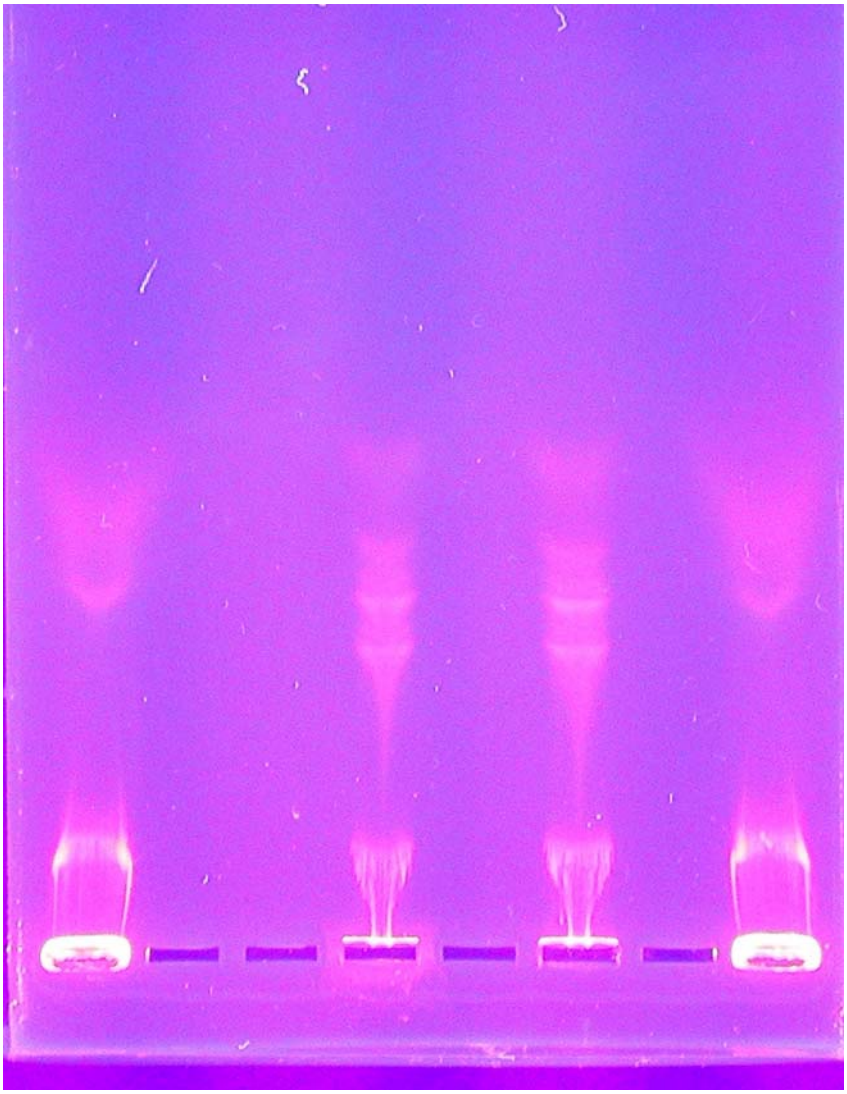

Figure no-7

The TLC of AcO2 strain

9. SUMMARY AND CONCLUSION

The total 6 Actinomycete strains were recovered from the soil sediment samples which were collected from regions of Coimbatore, Erode, Salem, Nagai districts of Tamil Nadu by using Glucose yeast extract malt agar medium, Inorganic salt agar medium and Luria-Bertani agar medium.

The cultural characteristics of the isolated strains were studied. Morphological and biochemical characteristics of the strains AcO1 to AcO6 were studied. The fermentation of the AcO1 to AcO6 was carried out by using Luria-Bertani broth, Inorganic salt broth and Glucose yeast extract malt broth. The fermentation was carried out on rotary shaker at 200rpm at 30ºC. After fermentation the recovery of the fermentation broth was carried by centrifugation and whatmann filtration.

Extraction of the supernatants S_2, S_6, S_{10}, S_{15}, S_{20}, S_{23} were carried out by using ethyl acetate, acetone and methanol. The methanol and acetone extract of AcO2 were recovered and their antiviral activity was studied by using Herpes simplex type-1.

The DNA of AcO2 was extracted by using cell lysis technique and purification was carried out by agarose gel electrophoresis. The DNA bands were separated according to ascending the molecular wt. as compare with standard marker DNA.

From the present work it is concluded that the soil of the region of Coimbatore, Erode, Salem, Nagai, district of Tamil Nadu have the capability to produced actinomycetes . The soil samples which were collected from Coimbatore showed potential for producing actinomycetes which are related to some species. The AcO2 was one of the strain related to *streptomyces* species. It is non-motile, non- acid fast from study. It was concluded that GLM medium is one of the best medium for isolation of actinomycetes from soils. Among the various actinomycetes strains isolated, the strain of AcO2 shows very good inhibition over the virus Herpes simplex virus type I. So this study used for developing a new drug against such type of virus, which may be very safe, less toxic and cheap when compared with other drugs.

The DNA obtained from AcO2 was free of contaminants like humic acid. Isoproponal which was preferred instead of poly ethylene glycol and ethanol. The analysis of the methanol and acetone extracts of AcO2 Rf values were found to be as 0.86 and 0.87 by thin layer chromatography. The λ max of extract AcO2 was found to be 232 nm. The functional group present in the extract was found from IR studies –C-H, C=N,C=O, C=C and aromatic disubstituted compound. The molecular weight of AcO2 was found peak at 189 is the base peak of most intense having 100% relative abundance. I am confident from the present study my work will render satisfactory results in future study

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APPENDIX

1. WAKSMAN MEDIA NO.42

Yeast extract	1.0 gm
L-tyrosine	1.0 gm
Sodium chloride	8.5 gm
Agar	16.0 gm
Distilled water	1000 ml
pH	6.8

2. ORGANIC NITRATE BROTH

Peptone	0.5 gm
Meat extract	0.3 gm
Potassium nitrate	0.1 gm
Distilled water	100 ml
pH	7.0

3. MEDIUM FOR HYDROGEN SULPHIDE

Ferric ammonium citrate	0.5 gm
Dibasic potassium phosphate	1.0 gm
Sodium thio sulphate	0.02 gm
Yeast extract	1.0 gm
Agar	20.0 gm
Distilled water	1000 ml
pH	7.0

4. GELATIN LIQUIFICATION MEDIA

Peptone	5.0 gm
Beef extract	3.0 gm
Gelatin	12.0 gm
Distilled water	1000 ml
pH	6.8

5. GLUCOSE NUTRIENT BROTH

Peptone	5.0 gm
Meat extract	3.0 gm
Glucose	10.0 gm
Distilled water	1000 ml
pH	7.0

6. LB MEDIA

Tyrosine	10.0 gm
Yeast extract	5.0 gm
Sodium chloride	2.0 gm
Agar	15.0 gm
Distilled water	1000 ml
pH	7.0

7. INORGANIC SALT AGAR MEDIA

Sodium carcinate	2.0 gm
L-asparginase	1.0 gm
Sodium propionate	4.0 gm
Dipotassium hydrogen phosphate	0.5 gm
Magnesium sulphate	0.1 gm
Ferrous sulphate	0.01 gm
Agar	15.0 gm
Distilled water	1000 ml
pH	7.0

8. GLM MEDIA

Yeast extract	3.0 gm
Malt extract	3.0 gm
Peptone	5.0 gm
Glucose	10.0 gm
Agar	15.0 gm
Distilled water	1000 ml
pH	6.8

9. LB BROTH

Tyrosine	10.0 gm
Yeast extract	5.0 gm
Sodium chloride	2.0 gm
Distilled water	1000 ml
pH	7.0

10. INORGANIC SALT BROTH

Sodium carcinate	2.0 gm
L-asparginase	1.0 gm
Sodium propionate	4.0 gm
Dipotassium hydrogen phosphate	0.5 gm
Magnesium sulphate	0.1 gm
Ferrous sulphate	0.01 gm
Distilled water	1000 ml
pH	7.0

11. GLM BROTH

Yeast extract	3.0 gm
Malt extract	3.0 gm
Peptone	5.0 gm
Glucose	10.0 gm
Distilled water	1000 ml
pH	6.8